Prevalence of virulence genes among clinical isolates of Pseudomonas aeruginosa collected from Peshawar, Pakistan
Kafeel Ahmad, Amjad Ali, Shaista Rahat

Abstract
Objective: To investigate six virulence genes in clinical isolates of pseudomonas aeruginosa collected from tertiary care hospitals.
Methods: The cross-sectional study was conducted from December 2014 to June 2016 at the Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan and comprised pseudomonas aeruginosa isolates collected from Khyber Teaching Hospital, Peshawar, and Hayatabad Medical Complex, Peshawar, Pakistan. The isolates were recovered from pus, urine, sputum, wound, bronchial wash, cerebrospinal fluid, blood, high vaginal swab and diabetic foot. Genomic deoxyribonucleic acid was extracted from the isolates after identification and polymerase chain reaction technique was performed for the molecular detection of six virulence genes using specific primers. The six genes were: algD, lasB, toxA, plcH, plcN, and exoS.
Results: There were 182 pseudomonas aeruginosa isolates. The prevalence of algD was 179(98.3%), toxA 156(85.7%), lasB 179(98.3%), plcH 178(97.8%), plcN 170(93.4%) and exoS 175(96.1%).
Conclusions: There was high prevalence of virulence factors among regional isolates of Pseudomonas aeruginosa.
Keywords: Pseudomonas aeruginosa, Virulence factors, Exotoxin A, Exoenzyme S, Elastase B. (JPMA 68: 1787; 2018)

Introduction
Pseudomonas (P.) aeruginosaisagram-negative bacterium that is responsible for a wide range of nosocomial infections. Owing to its resistant drug profile, the bacterium limits therapeutic options.1 P. Aeruginosa causes infections like pneumonia, urinary tract infection (UTI), meningitis, bacteraemia, otitis externa, endophthalmitis and endocarditis. Mostly, these infections occur in burns, cystic fibrosis and wounds patients. The infections could be more severe in immune-suppressed individuals like cancer and neutropenic patients.2 Various virulence factors are produced by P. aeruginosa which play a role in invasion and toxicity.3 P. aeruginosa produces virulence factors some of which are involved in acute infections and others in chronic infections. Factors contributing towards acute infection include exoenzyme S, exotoxin A, phospholipase C and pilli. Siderophores and pseudcapsule of alginate are the factors that help in protecting the bacterium from antibiotics, phagocytosis and dehydration.4 Elastase and type III secretion system contribute to pneumonia caused by P. aeruginosa.5 The exotoxin A of P. aeruginosa inhibits protein synthesis in eukaryotic cell. During this process, adenosine-5'-diphosphate-ribosyl (ADP-ribose) moiety of nicotinamide adenine dinucleotide (NAD) is transferred to eukaryotic elongation factor 2 (EF-2) which inactivates the EF-2 and ultimately inhibits protein synthesis.6,7 Exoenzyme S, coded by exoS gene, is an ADP-ribosyltransferase that modifies eukaryotic proteins by disturbing the Ras-mediated signal transduction pathway.8 Elastase B, coded by lasB gene, is a zinc metalloproteinase that destroys proteins involved in immune systems such as cytokines.9,10 The gene AlgD encodes alginate which is required for biofilm formation and protection from antibiotics and host immune response.11 Two types of phospholipase C (PLC) enzymes, the phospholipase C haemolytic (PLC-H) and the phospholipase C non-haemolytic (PLC-N), are produced by P. aeruginosa. PLC-H causes lysis of human erythrocytes and has the ability to degrade eukaryotic cell membrane sphingomyelin and phosphatidylcholine. PLC-N hydrolyses the membrane phospholipids phosphatidylserine and phosphatidylcholine.12 The current study was planned to report the occurrence of algD, lasB, toxA, plcH, plcN and exoS genes in regional isolates of P. aeruginosa.

Materials and Methods
The cross-sectional study was conducted from December 2014 to June 2016 at the Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan. The isolates were recovered from pus, urine, sputum, wound, bronchial wash, cerebrospinal fluid, blood, high vaginal swab and diabetic foot. Genomic deoxyribonucleic acid was extracted from the isolates after identification and polymerase chain reaction technique was performed for the molecular detection of six virulence genes using specific primers. The six genes were: algD, lasB, toxA, plcH, plcN, and exoS.

Results
There were 182 pseudomonas aeruginosa isolates. The prevalence of algD was 179(98.3%), toxA 156(85.7%), lasB 179(98.3%), plcH 178(97.8%), plcN 170(93.4%) and exoS 175(96.1%).

Conclusions
There was high prevalence of virulence factors among regional isolates of Pseudomonas aeruginosa.

Keywords: Pseudomonas aeruginosa, Virulence factors, Exotoxin A, Exoenzyme S, Elastase B. (JPMA 68: 1787; 2018)
Microbiology, University of Peshawar, Pakistan and comprised P. aeruginosa isolates collected from Khyber Teaching Hospital, Peshawar, and Hayatabad Medical Complex, Peshawar, Pakistan. The sample size was calculated in line with the overall prevalence of virulence factors and confidence interval of 95%. These included isolates from pus, urine, sputum, bronchial wash, diabetic foot, blood, high vaginal swab, wounds, and cerebrospinal fluid (CSF).

Informed consent was obtained from the patients and the study was approved by the institutional ethics committee.

The samples were inoculated onto MacConkey agar (Oxoid, UK) and incubated for 24 hours at 37°C. Isolates were identified using morphological (gram staining) and biochemical tests (catalase, oxidase, citrate utilisation test, triple sugar iron test, indole test and nitrate reduction test) according to standard procedures. Genomic deoxyribonucleic acid (DNA) was extracted using Gene JET Genomic DNA purification kit (Thermo Scientific, Lithuania) according to the kit protocol. Quality of DNA was analysed on 0.8% agarose gel. DNA was preserved at -20°C till further use.

The virulence genes — toxA, exoS, lasB, algD, plcH and plcN — were amplified using previously reported primers. The primers were prepared through Macrogen (Korea). A 20 μl polymerase chain reaction (PCR) volume consisted of 4 μl Master Mix (Solis BioDyne, Estonia, Cat. No. 04-12-00115) with 1 μl (0.5 μM) of each primer, 1 μl genomic DNA and 13 μl molecular biology grade water (Sigma-Aldrich, US). The amplification conditions consisted of an initial denaturation at 95°C for 5 min. Amplification of toxA was carried out using 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 1 min and extension at 72°C for 1 min. Amplification of lasB, algD, plcH and plcN were carried out using 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. All the reaction conditions included a final extension at 72°C for 5 min. All the genes were amplified using the PCR protocol reported in literature with slight modifications. The amplified products were analysed using 1.5% agarose gel. A 100 base pair (bp) DNA ladder (Bioron, Korea) was used for size comparison.

Results

Of the 182 P. aeruginosa isolates, 73 (40%) were from pus, 33 (18%) urine, 23 (12.6%) sputum, 11 (6%) bronchial wash, 3 (1.6%) diabetic foot, 6 (3.2%) blood, 5 (2.7%) high vaginal swab, 20 (11%) wounds, and 8 (4%) isolates were from CSF.

The prevalence of algD was 179 (98.3%), toxA 156 (85.7%), lasB 179 (98.3%), plcH 178 (97.8%), plcN 170 (93.4%) and exoS 175 (96.15%) (Table; Figures 1-6).

Table: Prevalence of virulence genes in Pseudomonas aeruginosa isolated from different clinical specimens.

<table>
<thead>
<tr>
<th>Specimen (number of isolates)</th>
<th>AlgD</th>
<th>lasB</th>
<th>toxA</th>
<th>plcH</th>
<th>plcN</th>
<th>exoS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pus (73)</td>
<td>73 (100%)</td>
<td>71 (97.26%)</td>
<td>61 (83.56%)</td>
<td>72 (98.63%)</td>
<td>69 (94.52%)</td>
<td>71 (97.26%)</td>
</tr>
<tr>
<td>Urine (33)</td>
<td>32 (96.96%)</td>
<td>32 (96.96%)</td>
<td>27 (81.81%)</td>
<td>33 (100%)</td>
<td>30 (90.90%)</td>
<td>32 (96.96%)</td>
</tr>
<tr>
<td>Sputum (23)</td>
<td>22 (95.65%)</td>
<td>23 (100%)</td>
<td>21 (91.3%)</td>
<td>22 (95.65%)</td>
<td>22 (95.65%)</td>
<td>23 (100%)</td>
</tr>
<tr>
<td>Wound (20)</td>
<td>20 (100%)</td>
<td>20 (100%)</td>
<td>17 (85%)</td>
<td>18 (90%)</td>
<td>18 (90%)</td>
<td>18 (90%)</td>
</tr>
<tr>
<td>Bronchial wash (11)</td>
<td>10 (90.9%)</td>
<td>11 (100%)</td>
<td>11 (100%)</td>
<td>11 (100%)</td>
<td>10 (90.9%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>Cerebrospinal fluid (8)</td>
<td>8 (100%)</td>
<td>8 (100%)</td>
<td>6 (75%)</td>
<td>8 (100%)</td>
<td>8 (100%)</td>
<td>7 (87.5%)</td>
</tr>
<tr>
<td>Blood (6)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>5 (83.33%)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>5 (83.33%)</td>
</tr>
<tr>
<td>High vaginal swab (5)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Diabetic foot (3)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>2 (66.66%)</td>
<td>3 (100%)</td>
</tr>
</tbody>
</table>

PCR: Polymerase chain reaction
DNA: Deoxyribonucleic acid.
P. aeruginosa produces a number of virulence factors which help in invasion and damage to host tissues. The expression of most of these virulence factors are regulated by two component transcriptional regulatory system and quorum sensing which are essential for the survival and growth of bacteria in the host. The most crucial virulence factors are alginate, elastase, exotoxin A, phospholipase and exoenzyme S which are regulated through proper signalling mechanisms. When the bacteria are high in number, P. aeruginosa produces two toxins elastase and pyocyanin via quorum sensing.

**Figure-2:** PCR amplification of lasB gene: lane L (100 bp DNA ladder); C (positive control); Lanes 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27 have lasB(284 bp) gene positive Pseudomonas aeruginosa samples.

**Figure-3:** PCR amplification of plcH gene: lane L (100 bp DNA ladder); C (positive control); Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27 have plcH (608 bp) gene positive Pseudomonas aeruginosa samples.

**Figure-4:** PCR amplification of exoS gene: lane L (100 bp DNA ladder); C (positive control); Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 have exoS (444 bp) gene positive Pseudomonas aeruginosa samples.

**Figure-5:** PCR amplification of plcN gene: lane L (100 bp DNA ladder); C (positive control); Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27 have plcN (481 bp) gene positive Pseudomonas aeruginosa samples.

**Figure-6:** PCR amplification of toxA gene: lane L (100 bp DNA ladder); C (positive control); Lanes 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26 have toxA (270 bp) gene positive Pseudomonas aeruginosa samples.

**Discussion**

P. aeruginosa produces a number of virulence factors which help in invasion and damage to host tissues. The expression of most of these virulence factors are regulated by two component transcriptional regulatory system and quorum sensing which are essential for the survival and growth of bacteria in the host. The most crucial virulence factors are alginate, elastase, exotoxin A, phospholipase and exoenzyme S which are regulated through proper signalling mechanisms. When the bacteria are high in number, P. aeruginosa produces two toxins elastase and pyocyanin via quorum sensing.
signalling mechanism.\(^{18}\) P. aeruginosa inject virulence factors exoS and exoU directly into cytosol of host cell through type III secretion (TTS) system mechanism.\(^{18}\) Current work showed that the prevalence of virulence genes algD, lasB were highest followed by plcH, exoS, plcN and toxA.

The algD gene was detected in all bacterial strains isolated from pus, wound, cerebrospinal fluid, blood, high vaginal swab and diabetic foot. Harsh environmental conditions and immune system of host causes stress on bacteria and make its survival difficult. P. aeruginosa produce extracellular polysaccharides like alginate, Psl, and Pel which protect bacteria from adversity in the environment. The alginate producing P. aeruginosa is often associated with isolates recovered from chronic pulmonary infection in cystic fibrotic patients.\(^{19}\) The in vitro production of alginate was observed among the isolates of P. aeruginosa recovered from urinary tract infection (UTI).\(^{20}\) The prevalence of lasB gene was observed in all strains isolated from sputum, wound, bronchial wash, cerebrospinal fluid, blood, high vaginal swab and diabetic foot. The elastase lasB has a role in the degradation of host collagen and non-collagen proteins, thus damaging the physical barrier of host and facilitating the dissemination of infection.\(^{21}\) High-level production of elastase was detected in vitro among most of P. aeruginosa isolated from urinary tract, trachea and wound.\(^{22}\) Prevalence of these has been reported from different parts of the world. A study from Poland reported 90.9% prevalence of algD and lasB gene in isolates from urine while the two genes were present in all the isolates from wound and bronchial specimens.\(^{15}\) The frequency of toxA gene was 81.8% in isolates from urine and 100% in isolates from wound and bronchial wash.\(^{15}\) Current work also showed similar frequencies of toxA gene in isolates from urine (81.8 %) and wound (85%) and bronchial wash (100%). P. aeruginosa toxin can inhibit protein synthesis.\(^{6}\) Under in vitro conditions higher level production of toxin A was observed in P. aeruginosa isolates obtained from wounds.\(^{22}\) Alginate plays an essential role in protecting biofilm forming P. aeruginosa from human immune response.\(^{23}\) The elastase enzyme damages tissues and degrades plasma proteins such as complement factors and immunoglobulins which are vital for the immune system of host.\(^{24}\)

A high frequency of plcH, plcN and exoS was observed in the current study. High-level production of phospholipase C was noted in vitro among most of P. aeruginosa isolates recovered from urinary tract, trachea and wound.\(^{23}\) However, rare production of phospholipase C was observed in vitro by Tielen et al.\(^{20}\) The virulence factor exoS is a bifunctional toxin with both adenosine diphosphate ribosyl transferase (ADPRT) activity and guanosine triphosphate-ase (GTPase) activating protein (GAP) activity.\(^{21}\) The adverse effect of ADPRT activity of exoS on host cell includes cell death, disruption of actin cytoskeletal and inhibition of DNA synthesis.\(^{21}\) The GAP activity is required for the anti-phagocytic effect of exoS.\(^{25}\) The in vitro analysis of virulence factors of P. aeruginosa showed high cytotoxic activity for exoU whereas low and intermediate cytotoxic activity was reported for exoS.\(^{20}\)

A study from Iran also reported higher frequencies of plcH (87.7%), plcN (60%) and exoS (70.8%)in isolates from cystic fibrosis patients while prevalence of these genes in isolates from burn specimens was 79%, 63.1% and 21.1% respectively.\(^{26}\) Another study from Egypt reported a low prevalence (10%) of exoS from blood samples.\(^{27}\) High prevalence of plcH (75%) in isolates from UTIs was reported from India.\(^{28}\) The virulence factors PLC-H and PLC-N act cooperatively and break down phospholipids which have cytotoxic effects on lymphocytes and neutrophils.\(^{29}\)

A very high prevalence of toxA and lasB gene was observed in this study. Previously, a study reported low prevalence (20%) of toxA and high prevalence (100%) of lasB gene in isolates from blood collected from Egyptian hospitals.\(^{21}\) High prevalence of toxA (100%) and lasB (75%) was reported from India in isolates from UTIs.\(^{28}\)

The limitation of the current study was the absence of colonisers or non-pathogenic strains among the collected isolates. Inclusion of colonisers could help in comparison of resistance genes prevalent among pathogenic strains and colonisers. The occurrence of resistance genes among pathogenic strains is clinically more important as they could limit treatment options by providing resistance against routinely used antibiotics.

To the best of our knowledge, this is the first study on the subject from this region. As each of these virulence factors contributes to the pathogenicity of P. aeruginosa, such high frequencies are alarming and need immediate action and proper management in order to reduce and finally eliminate this health-related issue.

**Conclusion**

The distribution of virulence genes algD, lasB, toxA, plcH, plcN and exoS was remarkably high in clinical isolates of P. aeruginosa isolated from pus, urine, sputum, wound, bronchial wash, cerebrospinal fluid, blood, high vaginal swab and diabetic foot.

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Conflict of Interest: None.

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References